The University of Chicago

TISSUE IMMUNITY

A DISSERTATION SUBMITTED TO THE
GRADUATE FACULTY IN CANDIDACY
FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF PATHOLOGY

By
GUILLERMO ALFREDO PACHECO R.

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LOCAL TISSUE IMMUNITY

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Immunologic investigations of recent years place increasing emphasis on the direct part played by the mesenchymal tissues in resistance to infectious diseases, and as a result cellular reactions of inflammation are being studied with renewed interest in the attempt to understand their fundamental significance in the general mechanism of defense. The idea of localization of infection as a definite defensive factor has assumed greater importance in the minds of many students of the problem, although the importance of general humoral elements is not denied. The conception has developed that increased resistance to certain infectious agents may be largely local, due to a more effective response of local tissues to the invading micro-organism. This notion, while not new, has been emphasized particularly by Besredka,[1] who has stimulated renewed interest in the problem of local immunity. New facts have resulted from this interest which not only strengthen the view that such an immunity actually exists, but also help to explain its mechanism.

In this paper experiments are reported which further support the idea that local immunity can be acquired.

There is at present some confusion regarding what is meant by the term local immunity. Besredka,[2] for example, defined it as "an immunity without the obligatory participation of antibodies." Gay,[3] on the other hand, defined the condition as a "locally superior mechanism for the disposal of a particular micro-organism," and stated that it may be demonstrated "either by the local presence of antibodies before their appearance elsewhere in the body, by their local presence in greater concentration than elsewhere, or by a superior method of direct disposal of bacteria in the particular area in question." Opie [4] interpreted local immunity after a previous sensitization to a specific antigen or bacterium as a local hypersensitivity that is manifested by an acute inflammation having the essential features of a local anaphylactic reaction, and tending to retard the entrance of bacteria or their noxious products

From the Department of Pathology and the Douglas Smith Foundation for Medical Research of the University of Chicago.

1. Besredka, A.: Compt. rend. Soc. de biol. **88**:1273, 1923.

2. Besredka, A.: Local Immunization, Baltimore, Williams & Wilkins Company, 1927.

3. Gay, F. P., in Jordan, E. O., and Falk, I. S.: The Newer Knowledge of Bacteriology and Immunology, Chicago, University of Chicago Press, 1928.

4. Opie, E. L.: J. Immunol. **17**:329, 1929.

into the blood stream. This retardation, he assumed, is brought about by the local accumulation of antibodies, which fix the antigen or bacteria at the site of inoculation, thus localizing the infectious agent and preventing its dissemination to more vital organs.

Regardless of differences in point of view concerning the actual mechanism of local immunity, most of the workers agree as to the existence of a localized condition that brings about an increased resistance to a specific micro-organism. This does not necessarily eliminate or minimize the importance of a generalized response.

HISTORICAL EVIDENCE FOR THE EXISTENCE OF LOCAL IMMUNITY

Fehleisen [5] in 1883 was probably the first to note evidence of experimental local immunity. He observed a locally increased resistance while reproducing erysipelas in animals and human beings who had recovered from previous attacks of the disease. Meierowitsch [6] in 1888 found that rabbits which had recovered from one attack of experimental erysipelas were protected from a second attack for from one to two months. As evidence that the immunity acquired after erysipelas is general as well as local, Roger [7] produced an attack of erysipelas in one ear of a rabbit, and after recovery had taken place, produced a second attack in the opposite ear, and found that it was followed by a rapid recovery or the formation of a small abscess. On the other hand, Gromakowsky [8] called attention to the absence of immunity against the streptococcus of erysipelas in the peritoneal cavity of animals previously immunized in the skin.

Cobbett and Melsome [9] produced both local and general immunity against erysipelas by subcutaneous inoculations of a pure culture of living streptococci into the ears of rabbits. They demonstrated the presence of local immunity by comparing the effects of inoculations of living streptococci into the ears of rabbits that had recovered from previous inoculations of the same micro-organism, with those of similar inoculations into normal rabbits. The response in the previously inoculated rabbits was manifested by an early local inflammatory reaction that subsided in from one to four days and the absence of general toxic effects. The control rabbits, on the other hand, presented a slow, progressive inflammatory reaction accompanied by general malaise and in many instances by death. The existence of a generalized immunity was demonstrated in two ways, first by producing an attack of erysipelas in the right ear and a few days after recovery producing a second attack in the left ear. The reaction was compared in extent and out-

5. Fehleisen: Aetiologie der Erysepelas, Berlin, T. Fischer, 1883.
6. Meierowitsch: Centralbl. f. Bakt. (ref.) **3**:406, 1888.
7. Roger, G. H.: Compt. rend. Soc. de biol. **42**:573, 1890.
8. Gromakowsky, D.: Ann. Inst. Pasteur **9**:621, 1895.
9. Cobbett, L., and Melsome, W. S.: J. Path. & Bact. **3**:39, 1896.

come with that of normal rabbits which had simultaneously been given the same quantity of streptococci. In half of the reinfected rabbits there were evidences of general immunity manifested as an early severe inflammatory reaction, which subsided in from three to four days. In a few instances, too, there were small abscesses as the end-results of the second attack of erysipelas. The second method was to inject streptococci into the peritoneal cavity and after recovery of the rabbit from this inoculation, to produce erysipelas in one ear. The course of the infection indicated that the rabbit had acquired an excellent general immunity, inferior to the local but superior to the general immunity produced by the inoculation of the opposite ear.

These investigators concluded that both local and general immunity resulted from the early inflammatory response, enhanced by the previous sensitization of the tissues. The lack of protection in the normal animals was interpreted as being due to the delay of the inflammatory reaction, which allowed the micro-organisms to multiply and produce their toxic products, which were not overcome by the phagocytes of the subsequent inflammatory reaction.

Römer [10] produced a local corneal immunity in the conjunctiva of one eye in rabbits by injecting small quantities of abrin. This local immunity he demonstrated by the resistance that the previously injected conjunctiva offered to further inoculation of abrin, while the opposite conjunctiva remained susceptible.

Noguchi [11] demonstrated a locally increased resistance to tetanus toxin in rats in which wounds were sutured with silk threads containing tetanus spores and treated with eosin, since the threads, when removed and transplanted in other rats or in the opposite sides of the same rats, caused fatal tetanus. He further showed that the leg previously resistant to the tetanus spores reacted very slowly to a subsequent inoculation of tetanus bacilli, whereas in the opposite leg or in normal rats the same quantity of bacilli produced a rapidly fatal outcome. Noguchi explained this local immunity as the result of a possible formation of antitoxins in the local connective tissues.

In 1905 Wassermann and Citron,[12] in testing the resistance to typhoid bacilli of animals immunized in the pleural cavity, peritoneal cavity and blood stream, noticed that the immunization was more marked if the bacilli were injected through the route previously used, and that this immunity was not dependent on the quantity of antibodies present in the blood serum. To explain this locally heightened resistance they postulated the idea of "Umstimmung," or retuning, of the local cells to an increased phagocytic activity.

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10. Römer, P.: Arch. f. Ophth. **52**:72, 1901.

11. Noguchi, H.: J. Exper. Med. **9**:291, 1907.

12. Wassermann, A., and Citron, J.: Ztschr. f. Hyg. u. Infektionskr. **50**:331, 1905.

Later, Wassermann and Ledermann [13] advised the application of killed cultures of staphylococci on infected wounds to prevent the development of complications. Similar observations were made by Levaditi,[14] who noticed that war wounds infected with streptococci quickly destroyed streptococci that were applied on dressings during the healing stage.

More recently, interest in the problem of local immunity has been stimulated by Besredka,[15] who in 1921 advanced a new theory to explain its mechanism. In this he suggested that local immunity is essentially a desensitization of certain receptive cells, which when immunized, become indifferent to specific micro-organisms, and allow them to be destroyed by the phagocytes. Although not definite as to where these receptive cells are located, he mentioned the reticulo-endothelial system as a possible location or source. He stated, furthermore, that this local immunity can be produced artificially by substances that contain the hypothetic agent, which he calls antivirus.[16] The application of this antivirus on or near the receptive cells produces an acquired local immunity without the intervention of antibodies. When the infection is present, this same antivirus acts as an immune serum in passive immunity, or directly produces in the tissues of the animal a medium in which the micro-organisms are incapable of multiplying, either because of desensitization of the receptive cells or because of a direct action on the bacteria present. To substantiate his ideas, the author described experimental work in animals and many clinical cases in which the antivirus had been used with favorable results.

In this country, Gay [17] has done much to establish local immunity as a definite entity. His work began with the production of experimental empyema in rabbits by intrapleural injections of a particular strain of streptococcus hemolyticus. He noticed under such conditions that the infection remained entirely local, although a general immunity was produced, as shown by the presence of agglutinins, opsonins and precipitins in the serum. The latter were demonstrable, however, only by a special serologic technic. Of more significance was his demonstration of the local morphologic changes produced in the pleural cavities of rabbits by the inoculation of various substances that stimulate definite cellular reactions, and the relation of these to the immunity which some of the rabbits had acquired.[18] From such experiments Gay concluded that the exudates containing large numbers of polymorphonuclear leukocytes were not protective when a minimal lethal dose was

13. Wassermann, A., and Ledermann, R.: Med. Klin. **7**:479, 1911.
14. Levaditi, C.: Compt. rend. Soc. de biol. **81**:1059, 1918.
15. Besredka, A.: Ann. Inst. Pasteur **35**:421, 1921.
16. Besredka, A.: Bull. Inst. Pasteur **28**:49 and 105, 1930.
17. Gay, F. P., and Stone, R. L.: J. Infect. Dis. **26**:265, 1920.
18. Gay, F. P., and Morrison, L. F.: J. Infect. Dis. **33**:338, 1923.

inoculated into the pleural cavity, whereas exudates containing larger numbers of clasmatocytes than polymorphonuclear leukocytes protected against as many as 200 M. L. D. of hemolytic streptococci. Therefore Gay concluded that the local acquired immunity was nonspecific and dependent on the high ratio of clasmatocytes to polymorphonuclear leukocytes. Later, however, he demonstrated that a more pronounced local pleural immunity could be secured when specific antibodies were added to the increased number of clasmatocytes.[19] Rivers and Tillett [20] showed also that protection against streptococci injected into the skin was far greater when the skin had been infiltrated twenty-four hours before or at the same time with the immune serum, than after similar treatment with normal serum or beef broth. Opie [21] observed that an antigen persisted at the site of inoculation in animals previously sensitized to that antigen, and suggested that the accumulation is due to the local presence of antibodies, which unite and fix the antigen at the site of inoculation.

Freedlander and Toomey [22] produced a nonspecific local immunity against Staphylococcus aureus in the abdominal walls of guinea-pigs, by the application of wet compresses of broth, mustard, saline solution, meat, peptone and 10 per cent horse serum. Their morphologic observations demonstrated an increased number of clasmatocytes in the subcutaneous tissues.

In summary, then, one may conclude that local immunity in varying degrees has been demonstrated in erysipelas, in experimental streptococcal empyema, during the process of healing of wounds, after nonspecific stimulation with foreign proteins and other substances that bring about an inflammatory process, after injection of killed cultures of streptococci and staphylococci into local areas of the skin and after inoculation of tetanus bacilli into wounds. In all of these cases the immunity has been either a combined local and general immunity, or a general immunity manifested in a local area by a heightened, rapid inflammatory response after a nonspecific stimulation of the local tissues, or a local immunity with a general immunity of lesser degree in other portions of the body. The presence of antibodies in the blood serum has been demonstrated in many instances, and serums have been prepared that increased the local defensive powers. In explaining the mechanism of local immunity, some have postulated a defensive system that is nonspecific and dependent only on the number of phagocytes; others, that the local area contains a greater concentration of antibodies, which when combined with the antigen bring about a positive chemotaxis. Practically all of the investigators of this field, however, agree that an early

19. Gay, F. P., and Clark, A. R.: J. Exper. Med. **52**:95, 1930.
20. Rivers, T. M., and Tillett, W. S.: J. Exper. Med. **41**:185, 1925.
21. Opie, E. L.: J. Immunol. **8**:55, 1923.
22. Freedlander, S. O., and Toomey, J. A.: J. Exper. Med. **47**:663, 1928.

inflammatory reaction and an increased phagocytic activity are constantly present.

THE EXPERIMENTAL PROBLEM

As can be seen, the problem of local immunity is still obscure, first as to its existence, second as to its independence from a general immunity and third as to the mechanism determining it. With these considerations in mind the following questions were formulated:

1. Is there such a thing as local immunity independent from general immunity?
2. If so, what is the mechanism?
3. How can this be demonstrated experimentally?

The following experiments were undertaken in the attempt to answer these questions:

1. A histologic study of the changes in the skin of normal guinea-pigs during the process of localized intradermal injections of killed cultures of S. aureus.
2. A comparison of the gross reactions of the skin to inoculations of a living, virulent culture of S. aureus in normal guinea-pigs, in those given previous intradermal injections of 0.85 per cent salt solution and those given local intradermal injections of a staphylococcal vaccine.
3. Observation of the changes and healing processes in the skin of the abdominal wall after excision of the area inoculated with a living, virulent culture of S. aureus in normal guinea-pigs and in those previously treated locally with a staphylococcal vaccine.
4. A study of the histologic changes occurring at hourly intervals from the fifth to the twenty-fourth hour after intradermal injection of a living, virulent culture of S. aureus into the skin of the abdominal walls of normal guinea-pigs, of those previously given intradermal injections of sterile salt solution and of those previously treated intradermally with staphylococcal vaccine.

MATERIALS AND METHODS

In these experiments, guinea-pigs weighing from 150 to 350 Gm. were used. The material used for inoculation was from a culture of S. aureus obtained from a large furuncle on the upper lip of a patient who had suffered from fever, chills and malaise of ten days' duration. The virulence of the organism was determined by inoculating guinea-pigs intradermally in the abdominal wall with 0.2 cc. of a twenty-four hour agar culture, diluted in 1 cc. of physiologic solution of sodium chloride (0.85 per cent NaCl). The necrosis of an area about 0.5 cm. in diameter or the death of the guinea-pig within ninety-six hours was taken as a criterion of virulence.

Three methods of inoculation were tried to see which would cause the most pronounced inflammatory reaction: viz., with the bacteria diluted in 0.85 per cent solution of sodium chloride; with the bacteria in a 1:500 solution of calcium

chloride, and with the organisms suspended in melted agar at from 45 to 50 C. Of these three methods, the first was found easiest to control; it gave as good results as the others and did not introduce complicating reactions; it was therefore adopted as a routine procedure. A twenty-four hour agar culture of S. aureus was suspended in 1 cc. of sterile salt solution and heated at 60 C. for one hour, after which the suspension was transferred to a tuberculin hypodermic syringe equipped with a no. 28 gage needle, and 0.2 cc. was inoculated intradermally into the right side of the previously prepared anterior abdominal wall. An area measuring about 4 by 2 cm. had been shaved, carefully washed with tap water and soap, rinsed with sterile water and finally painted with a weak solution of iodine in a mixture of equal parts of ether and alcohol.

In all, ten daily injections, about 1 cm. apart, were made, the first three into the right anterior abdominal wall, the next four into the midline and the last three into the left abdominal wall. Control animals were similarly given injections of 0.2 cc. of 0.85 per cent solution of sodium chloride. The guinea-pigs were then left undisturbed for twenty-five days, after which they, with normal animals, were inoculated with 0.2 cc. of a twenty-four hour growth of living S. aureus diluted in 1 cc. of sterile salt solution.

Histologic study was made of pieces of tissue cut from the inoculated areas after they had been fastened with bamboo pegs on square frames of cork as advised by W. Bloom,[23] fixed in Zenker's fluid plus solution of formaldehyde, embedded in celloidin, cut at from 10 to 15 microns and stained with hematoxylin, eosin and azure II (method of Maximow[24]). For the study of the bacteria, Gram's and Claudius'[25] staining methods were used on smears and sections. For the determination of fibrin, the specific stains of Weigert,[26] Kockel,[27] Wolff,[28] Unna,[29] Schmidt[30] and Wallace[31] were used. In staining for fibrin, sections of normal and previously inoculated skins during different hours of the inflammatory process were stained, with an equal number of sections of pneumonic lung that contained fibrin. The latter served as controls on the efficiency of the stains at the time they were used.

GROSS AND HISTOLOGIC CHANGES PRODUCED IN THE SKIN OF NORMAL GUINEA-PIGS BY TEN DAILY INTRACUTANEOUS INJECTIONS OF STAPHYLOCOCCAL VACCINE

As a preliminary step, the effects of the daily intracutaneous injections of the heat-killed staphylococcal vaccine were first observed in twelve guinea-pigs treated as follows:

On the first day, all of the guinea-pigs were given an intradermal injection of 0.2 cc. of the vaccine. All, except the one that was to be sacrificed twenty-four

23. Bloom, W.: Personal communication.
24. Maximow, A.: Ztschr. f. wissensch. Mikr. **26**:177, 1909.
25. Claudius, M.: Ann. Inst. Pasteur **11**:332, 1897.
26. Weigert, C.: Fortschr. d. Med. **5**:228, 1887.
27. Kockel: Centralbl. f. allg. Path. u. path. Anat. **10**:749, 1899.
28. Wolff, E.: Ztschr. f. wissensch. Mikr. **15**:310, 1898-1899.
29. Unna, P. G.: Monatschr. f. prakt. Dermat. **20**:140, 1895.
30. Schmidt, H.: Beitr. z. Anat., Physiol., Path. u. Therap. d. Ohres **27**:455, 1929.
31. Wallace, Helene M.: Science **74**:369, 1931.

hours later, were vaccinated in the right upper quadrant. In the latter the injection was made in the middle of the space between the umbilicus and the lower border of the sternum. This was done to study the same histologic structure in each animal, altered only by the effects of the vaccination. Twenty-four hours later the guinea-pig vaccinated in the center of the abdominal wall was killed after the area receiving the injection had been fixed on a cork frame. The same procedure was followed in the remaining animals in order to obtain ten specimens of skin that would demonstrate the progressive daily histologic changes occurring during the process of injections.

Gross Changes.—The gross changes occurring in the skin of normal guinea-pigs following the first injection of the staphylococcal vaccine were either the formation of a hyperemic nodule, varying from 2 to 10 mm. in diameter, and tending to become firmer and finally disappearing but leaving a pigmented macule, or the development of a nodule that softened, ulcerated, discharged a thick, yellow purulent material and then underwent rapid healing, leaving a slightly pigmented scar.

The subsequent injections usually produced larger nodules and produced them more rapidly, with more intensive reaction at the periphery and the base of the nodule. In many instances this reaction was so violent as to produce large areas of necrosis surrounded by elevated, hyperemic and edematous borders. The nodules of the previous injections were often reactivated to an inflammatory state by the later injections, as shown by the increased size of the area of hyperemia or by the formation of a necrotic area at the point of healing or in a quiescent nodule. In many guinea-pigs after the sixth injection a sudden rapid healing occurred in the previously elevated and hyperemic nodules, the discharge of some of the pustules disappeared and the entire skin was clean. In a few instances the violent local reaction after the fourth injection resembled grossly the Arthus phenomenon in the rabbit.

Histologic Changes.—The first injection of vaccine produced an inflammatory reaction characterized by dilatation of the lymphatics and blood vessels, edema, diapedesis of leukocytes, hemorrhage and moderate phagocytosis of the particles of vaccine by polymorphonuclear leukocytes and a small type of mononuclear cell. During the following injections, the inflammatory reaction was more intense, and there were signs of mobilization of the undifferentiated perivascular mesenchymal cells and of the connective tissue histiocytes and other reticular cells, with the appearance of numerous actively phagocytic mononuclear cells. In the first injection, the particles of vaccine were disseminated throughout the entire section, both extracellularly and in polymorphonuclear leukocytes. In the succeeding ones, the particles tended to localize within a relatively small area, which contained many polymorphonuclear leukocytes and was surrounded by many mesenchymal cells. After the second injection, the area of greatest reaction was that of the reticular and subreticular layers with a moderate cellular infiltration in the papillary and muscular layers.

In the reticular and subreticular layers, the predominant cells during the first injection were the polymorphonuclear leukocytes, but in the subsequent ones mononuclear cells also appeared in great numbers. The polymorphonuclear leukocytes were vacuolated, many showed pyknosis and karyorrhexis, while others were digested or were in the process of being digested by small and large mononuclear cells and elongated histiocytes.

The final result of the injections was a definite increase in the thickness of the dermis with increased proliferation of mesenchymal cells as

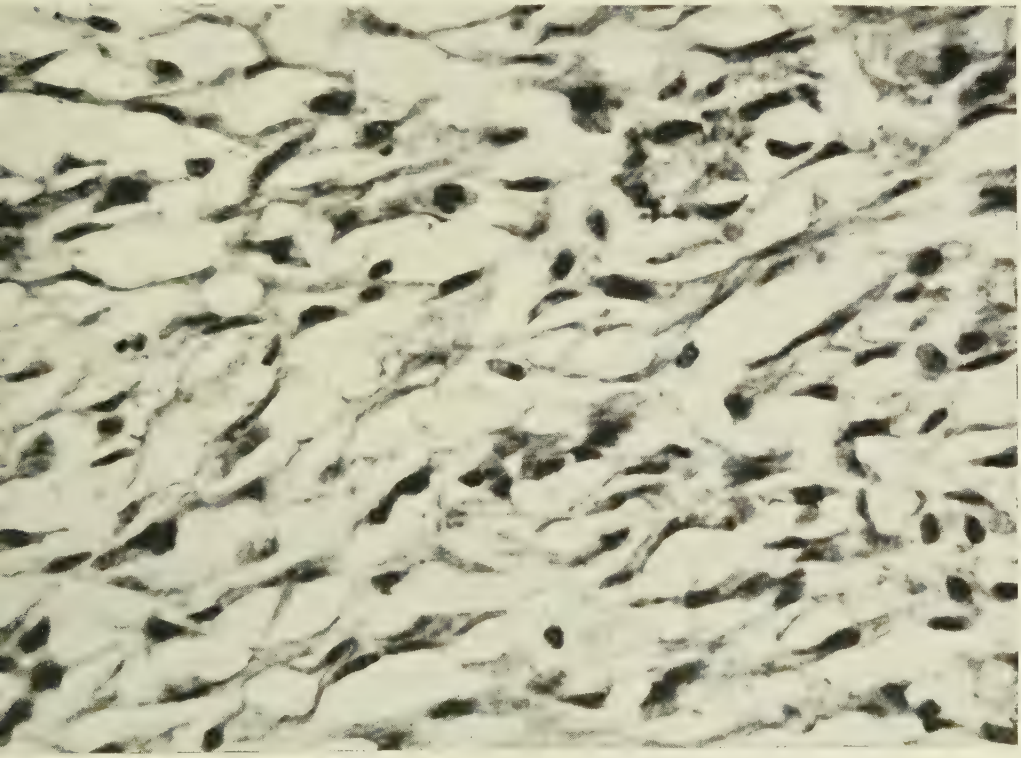

Fig. 1.—Photomicrograph (reduced from a magnification of × 700) of the subcutis of a guinea-pig after the tenth injection of the staphylococcal vaccine. Note the syncytium-like mass of mesenchymal tissue in which are many mitotic figures and some histiocytes with a foamlike cytoplasm.

manifested by the number of mitotic figures and infiltration by a great number of mesenchymal cells and leukocytes (fig. 1). The reticular and subreticular layers were greatly increased in thickness by innumerable quantities of mesenchymal and mononuclear cells, which were undergoing transformations resembling those seen in tissue cultures. There were rather numerous mitotic figures in the mesenchymal tissue and

pericytes of the blood vessels of the reticular, subreticular and muscular layers, and also a tendency toward the formation of a syncytium. Some of the mesenchymal cells were loaded with fat vacuoles. The polymorphonuclear leukocytes tended to disappear, leaving traces of their presence in the phagocytosed particles that were retained in the cytoplasm of the large mononuclear and histiocytic cells. The muscle layers were separated by a dense mesenchymal structure, which contained all the cell types of this tissue. The muscle bundles were separated by thick bundles of connective tissue. The blood vessels and lymphatics in most of the sections were dilated, patent and surrounded by many layers of undifferentiated pericytes, which frequently contained mitotic figures. In some instances the endothelial cells of the lymphatics and blood vessels were swollen. In the reticular and subreticular layers there was an increased number of new lymphatics and blood vessels. The entire thickness of the immunized skin was from three to ten times that of normal guinea-pigs.

GROSS CHANGES IN THE SKIN OF THE ABDOMINAL WALL IN GUINEA-PIGS AFTER INOCULATION OF LIVING, VIRULENT S. AUREUS

Normal Guinea-Pigs.—The gross changes produced in the skin and general health of normal guinea-pigs by the inoculation of 0.2 cc. of a living, virulent twenty-four hour agar culture of S. aureus were characterized, in general, by three types of reaction:

1. The formation in a few guinea-pigs of a small elevation of the skin with hyperemia and no further change during the entire period of observation.

2. The formation in some guinea-pigs of a hyperemic nodule which increased in size and density for from one to five days, until the surface became necrotic and opened to form a large, deep wound, which discharged pus and portions of the necrotic tissue for from twelve to sixteen days or more. Some of these guinea-pigs died before the wound was healed; in a few, secondary abscesses at the edges of the wound developed during the process of healing.

3. In others, an extensive cellulitis with the skin becoming purplish and edematous over nearly the entire anterior abdominal wall. The abdominal wall was cold, the guinea-pigs became extremely ill, and the majority of them died within from ten to forty-eight hours. From some of these, when the skin was cut at autopsy, much serosanguineous fluid escaped. The peritoneal surfaces were rough, with the fluid slightly turbid and increased in quantity, indicating peritonitis, presumably by extension from the area of cellulitis.

The gross effects in the twenty normal guinea-pigs inoculated with living staphylococci are summarized in table 1.

Guinea-Pigs Previously Given Intradermal Injections of Sterile Salt Solution.—The results obtained by the inoculation of 0.2 cc. of the living staphylococci into the guinea-pigs previously given intradermal injections of physiologic solution of sodium chloride were similar to those in the normal guinea-pigs, the gross effects being summarized in table 2.

It seems apparent, therefore, that repeated intradermal injections of salt solution did not modify in any significant way the reactivity of the tissues of the skin of the guinea-pigs to the subsequent injections of living staphylococci.

Guinea-Pigs Previously Given Local Injections of Staphylococcal Vaccine.—The results of the inoculation of the living staphylococci into

TABLE 1.—*Gross Effects on Normal Guinea-Pigs Inoculated Intradermally with Living S. Aureus*

Effect	Number	Per-centage
Death within ten days	6	30
No change	2	10
Complete healing after sixteen days	1	5
Healing wound but covered by a crust after sixteen days	5	25
Open wound after sixteen days	6	30

TABLE 2.—*Gross Effects of Intradermal Inoculation of Living S. Aureus in Guinea-Pigs Previously Given Intradermal Injections of Physiologic Sodium Chloride*

Effect	Number	Per-centage
Death within ten days	6	30
No change	1	5
Complete healing after sixteen days	1	5
Healing wound but covered by a crust after sixteen days	2	10
Open wound after sixteen days	10	50

the skin of guinea-pigs previously given local injections of the staphylococcal vaccine were as follows: In one guinea-pig a small, firm nodule appeared, which lasted for a few days and then gradually regressed. In others, small pustules developed, which discharged a thick, yellow purulent material and healed in from seven to fourteen days, leaving slightly pigmented scars. In most of the animals, however, nodules developed which increased in size and ulcerated after from three to five days, forming wounds that healed with small scars in fifteen days or less. It was apparent that these reacted quickly and effectively with rapid healing; furthermore, the death rate was one-half that of the normal guinea-pigs and that of the guinea-pigs previously inoculated with sterile physiologic solution of sodium chloride. The results for this group of twenty animals are summarized in table 3.

These experiments demonstrated that there is a great variability in the reactivity of the skin of normal guinea-pigs to the inoculation of living S. aureus. This may be explained by variation in natural immunity, which is more marked in some animals than in others. The results in the guinea-pigs given previous local injections of the staphylococcal vaccine indicated that the injection of killed staphylococcal vaccine had produced a change in the reactivity of the inoculated tissues to the extent of preserving the life of some guinea-pigs and of accelerating the healing process and restricting the dissemination of the bacteria in others.

TABLE 3.—*Gross Effects of Intradermal Inoculation of Living S. Aureus into Guinea-Pigs Previously Given Intradermal Injections of Staphylococcal Vaccine*

Effect	Number	Percentage
Death within ten days	3	15
No change	1	5
Complete healing after fourteen days	14	70
Healing wound but covered by a crust after sixteen days	1	5
Open wound after sixteen days	1	5

TABLE 4.—*End-Results of Inoculation of Living S. Aureus in Normal Guinea-Pigs and Those Previously Given Injections of Staphylococcal Vaccine from Which Inoculated Areas Were Excised*

Normal			Vaccinated		
	Number	Percentage		Number	Percentage
Dead	4	40	Dead	0	0
Healed in 13 days	1	10	Healed in 8 days	2	20
Healed in 14 days	2	20	Healed in 9 days	3	30
Healed in 19 days	3	30	Healed in 12 days	5	50

EFFECTS OF EXCISION OF THE AREA OF INOCULATION AFTER INTRADERMAL INJECTION OF LIVING STAPHYLOCOCCI

The rapidity of dissemination of living bacteria from the inoculated area was tested in another series of twenty guinea-pigs, ten of which were normal and ten of which had been previously treated locally with the staphylococcal vaccine. The guinea-pigs were paired according to weight, and the inoculations were performed five minutes apart. At intervals of one, two, three, five, seven, nine, twelve, fifteen, eighteen and twenty-four hours, a pair of animals was anesthetized, and from each a piece of tissue 10 by 10 by 2 mm. was excised around the point of inoculation. The wounds were closed with two or three stitches of silk no. 2, and the gross changes subsequently determined. Table 4 summarizes the after-effects. In the normal animals, healing was slow;

all the wounds opened within from one to four days, and there was a purulent discharge for more than sixteen days. The area where the silk stitches were placed became necrotic, and large pieces of greenish tissue sloughed off. The fatality rate in this series of animals was 40 per cent. The healing time was between thirteen and nineteen days, and in many guinea-pigs secondary abscesses developed in the neighborhood of the wound.

In contrast to these findings, the healing of the wounds in the guinea-pigs previously given injections of the staphylococcal vaccine was rapid, in some instances the wounds remaining closed and healing by first intention. In others, although the wounds opened completely, healing occurred within from eight to twelve days. Furthermore, no deaths occurred in these animals, and no abscesses recurred.

These experiments demonstrated that the bacteria in the skin of normal guinea-pigs disseminated quickly through the surrounding tissue, so that the excision of the infected area at one hour or at twenty-four hours did not prevent the fatal outcome or prevent delay in the healing processes. In the guinea-pigs previously given injections of the stapyhlococcal vaccine the bacteria were considerably localized, as demonstrated by the fact that the removal of the infected area protected the life of 40 per cent of these guinea-pigs, and shortened the period of healing of the wounds. Furthermore, the rapidity of healing of these tissues by primary union was striking, indicating that the harmful effects of the staphylococci had been largely eliminated.

HISTOLOGIC CHANGES AFTER INTRADERMAL INOCULATION OF LIVING S. AUREUS IN THE ABDOMINAL WALL IN GUINEA-PIGS

Similar experiments were next performed in order to study histologically at hourly intervals after the fifth hour the changes produced in the abdominal wall following the intradermal inoculation of 0.2 cc. of a living twenty-four hour agar culture of S. aureus. Sixty guinea-pigs were used, twenty normal, twenty previously given intradermal injections of the sterile salt solution, and twenty locally inoculated in the abdominal wall with the staphylococcal vaccine. From these three sets of guinea-pigs, groups of three of approximately equal weight (one from each set) were selected. In each group the individual inoculations of the living staphylococci were done five minutes apart in order to allow time to fix the tissues when they were later removed, and yet have similar hourly periods. After the fifth hour a group of three guinea-pigs was sacrificed each hour for the next twenty-four hours. By this procedure tissues showing hourly histologic changes following the inoculation of living staphylococci were obtained from twenty groups of guinea-pigs.

The general histologic changes occurring under these conditions have been described and illustrated in a previous paper (Cannon and

Pacheco), where the histologic differences in the normal and immunized animals were demonstrated. In the former, the characteristic picture was an extensive edema of the skin with dilatation of the blood vessels and lymphatics, hemorrhage and moderate infiltration by granular leukocytes and by a lesser number of small mononuclear cells. Histiocytes were inconspicuous, and there was no evidence of activation of the mesenchymal tissues. Some of the muscle bundles were undergoing hyaline degeneration. The blood vessels were dilated and engorged with blood, and at times contained many leukocytes that were in active stages

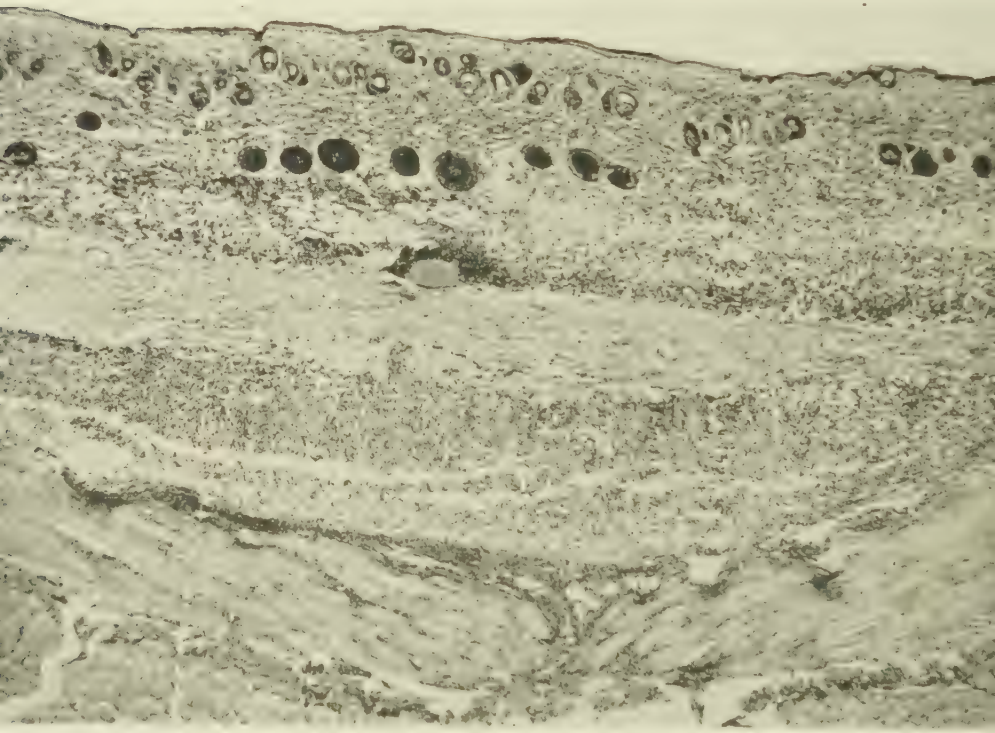

Fig. 2.—Photomicrograph (reduced from a magnification of × 70) of a section through the skin and entire abdominal wall of a normal guinea-pig inoculated with 0.2 cc. of a suspension of living, virulent S. aureus. The tissue was excised six hours after inoculation. The bacteria are disseminating through the reticulin or collagenic fibrils of the subreticular layer. There is a moderate cellular response, most of the cells being polymorphonuclear leukocytes, which contain many phagocytosed bacteria. The black area around the large venule consists of a concentrated mass of extracellular staphylococci, and the edematous layer of reticular fibrils beneath this contains large numbers of extracellular bacteria as shown in figure 3. The infection is obviously generalized, in spite of the abundant polymorphonuclear leukocytic response.

of diapedesis. The majority of the lymphatics were patent and dilated. No fibrin was found by the use of specific stains. The polymorphonuclear leukocytes showed innumerable bizarre figures, pyknosis, karyorrhexis and vacuoles. Enormous numbers of the polymorphonuclear leukocytes contained ingested staphylococci; many of the microorganisms were disseminated in long rows through the entire section, even extending into the connective tissue septums of the muscle layers. In many instances there were extensive areas of necrosis in the epi-

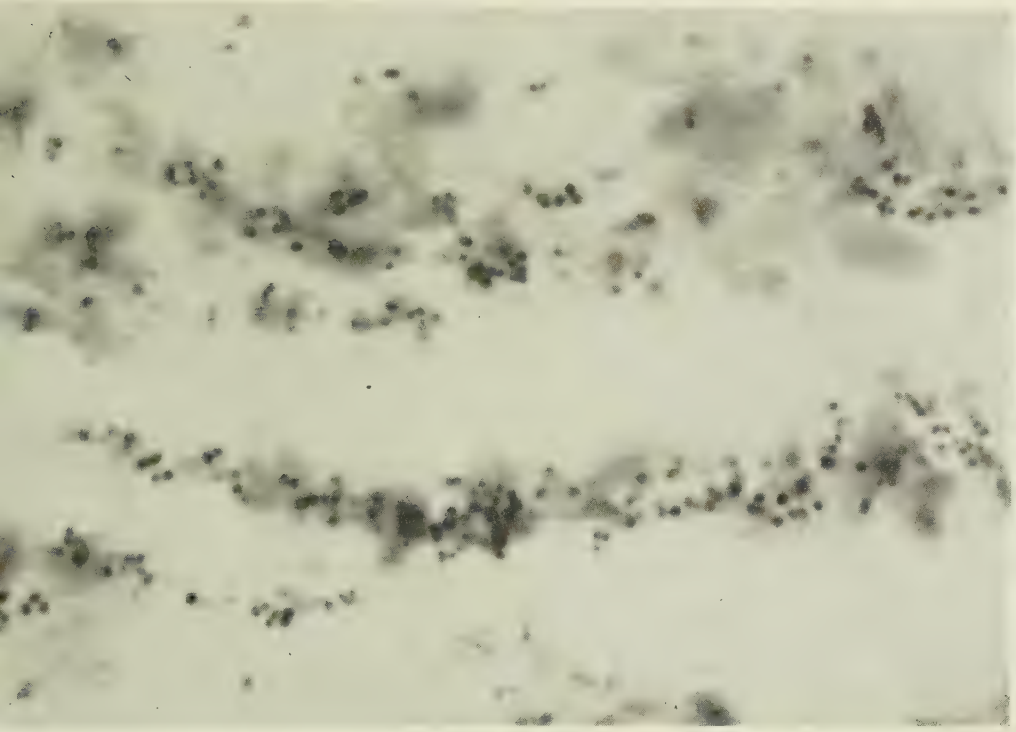

Fig. 3.—Photomicrograph (reduced from a magnification of × 2,650) of the section shown in figure 2. The bacteria are disseminating through the collagenic or reticulin fibrils. The cellular reaction is slight in this area. Note the absence of any agglomerative tendency of the bacteria.

dermis, papillary layers and muscularis. The subreticular layer was filled with polymorphonuclear leukocytes, eosin-stained precipitate and isolated groups or rows of free bacteria (fig. 2). The picture as a whole was that of an extensive cellulitis. It is noteworthy that in spite of widespread phagocytosis by the polymorphonuclear leukocytes the infection was disseminating widely.

The histologic changes in the skin of guinea-pigs previously given intradermal injections of normal salt solution were practically indistinguishable from those of the normal animals and will not be described in detail.

The histologic changes in the skin of guinea-pigs previously given local injections of the staphylococcal vaccine were characterized by a marked tendency to localization and clumping of the bacteria in a small area, which was filled with polymorphonuclear leukocytes, red blood

Fig. 4.—Photomicrograph (reduced from a magnification of × 70) of a section through the skin and a portion of the subreticular zone of a guinea-pig's abdominal wall previously immunized locally with the staphylococcal vaccine and later inoculated with 0.2 cc. of a living culture of S. aureus. Section taken six hours later. Note the localization of the bacteria in large masses in a small area, which is surrounded by a dense layer of mesenchymal cells. The area of polymorphonuclear infiltration and edema is very small. The black areas at the margins of the area of edema are masses of extracellular bacteria as shown in figure 5.

cells and small mononuclears (fig. 4). This localized area was in turn surrounded by large mononuclear leukocytes, histiocytes and many undifferentiated mesenchymal cells. As early as six hours, extracellular

masses of bacteria, as well as intensive phagocytosis by the small and large mononuclear and polymorphonuclear leukocytes, were noted. The polymorphonuclear response was intensive, but was localized largely to the area where the bacteria were fixed. Outside of the area, fewer polymorphonuclear leukocytes could be found between the mesenchymal tissues. The hemorrhage was also localized to the area where bacteria and the polymorphonuclear leukocytes were gathered, with the exception of two cases in which hemorrhage had occurred into the surrounding mes-

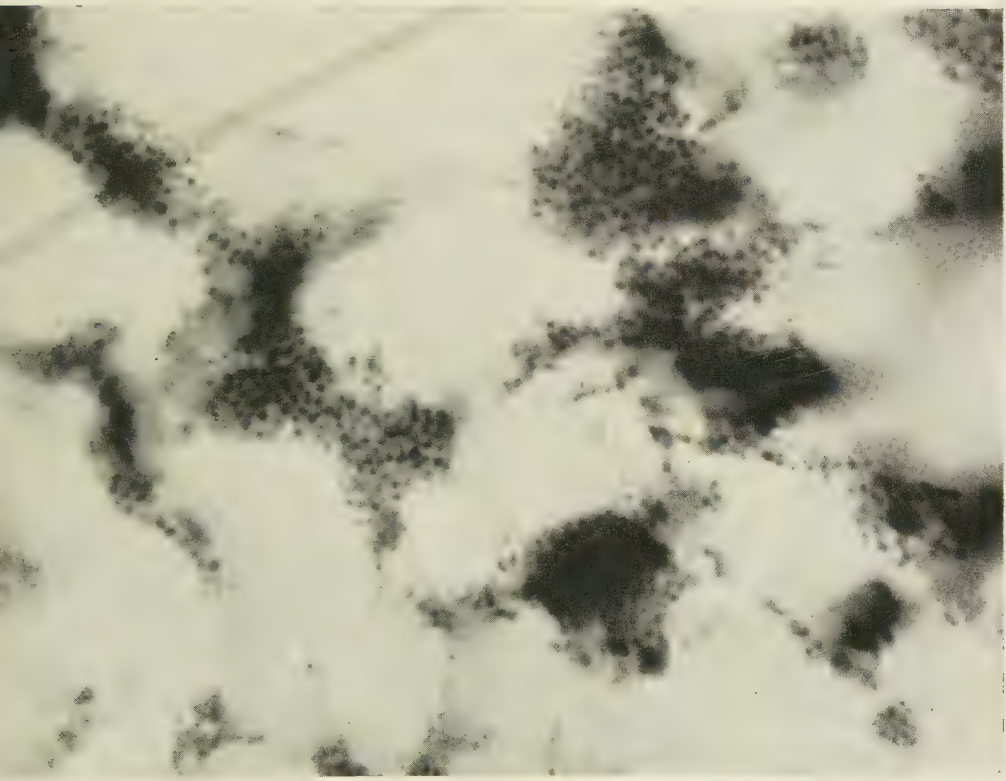

Fig. 5.—Photomicrograph (reduced from a magnification of × 2,650) of the section shown in figure 4 in an area where the bacteria are less concentrated. The bacteria are in masses. In many places the outlines of the bacteria cannot be made out.

enchymal tissue and the muscle layers. From the fifth to the twenty-fourth hour the polymorphonuclear leukocytes showed numerous bizarre shapes, were poorly stained, were vacuolated and showed evidences of degenerative changes. The mononuclear cells surrounding the polymorphonuclear leukocytes were in active stages of phagocytosis, as shown by the numbers of bacteria and polymorphonuclear leukocytes within their cytoplasm.

The bacteria as a whole were localized in large clumps to a small area as early as six hours after the inoculation (fig. 5), and after twelve hours most of them were intracellular. Both intracellular and extracellular injury of bacteria was indicated by their swollen and poorly stained appearance.

In the remainder of the reticular and subreticular layers there were several rows of connective tissue cells, many of which were proliferating, as shown by numerous mitotic figures. The area of localization was, as a rule, in the reticular and subreticular layers, although in a few cases the bacteria were localized in the papillary layer. In others there was a local necrosis of the epithelium and papillary layers. The papillary and muscular layers contained local phagocytic cells of many bizarre shapes, as though they had been fixed while in an active stage of ameboid movement, but very little necrosis was noted in these layers. The blood vessels and lymphatics were largely patent, and in many instances there were many patent new small blood vessels in the mesenchymal tissue of the subreticular layer. After a thorough search few thrombosed blood vessels or lymphatics were found; on the other hand, many of these structures seemed to be dilated. Fibrin could not be found with specific stains, and with hematoxylin and eosin the picture was that of a compact mass of bacteria, polymorphonuclear leukocytes and mononuclear cells, surrounded by a dense mass of mesenchymal tissue in which Mallory's connective tissue stain revealed a network of reticulin and collagenic fibrils in the reticular and subreticular layers.

COMMENT

These experiments demonstrate that living virulent staphylococci may be effectively localized in the cutaneous tissues of guinea-pigs previously given injections in the same area of a heat-killed suspension of S. aureus. This vaccination leads to the development of a locally superior mechanism for the disposal of living staphylococci when they are injected later. The experiments, however, do not differentiate between the effects of local and general resistance. Data concerning that problem will be presented in a later paper.

The specific purpose of this work was to study in detail the morphologic changes induced in the mesenchymal tissue by the injection of a staphylococcal vaccine, and to determine to what extent these changes altered the normal reactivity to a living, virulent suspension of S. aureus. Consequently, serologic studies of the blood serum and local tissues were not made.

It is apparent that the intradermal injection of heat-killed staphylococci called forth a pronounced cellular response in the skin of the anterior abdominal wall of guinea-pigs. This response was predominantly of polymorphonuclear leukocytes, followed shortly by mobilization

and proliferation of mononuclear cells. The latter arose *in situ* to a large extent, as shown by the numerous mitotic figures, but many also entered from the blood stream. By the fourth day these mononuclears were actively phagocytic and contained in their cytoplasm particles of vaccine and polymorphonuclear leukocytes in various stages of disintegration. By the tenth day of the inoculations the dermis was from three to ten times the thickness of that in normal animals. The reticular and subreticular layers looked like a syncytium, in which were many new blood vessels and lymphatics. The picture in general resembled that described by Gay, Clark and Linton [32] in the pleural and peritoneal wall of rabbits into which nonspecific irritants, such as aleuronat-starch mixtures, had been injected.

The inoculation of living, virulent staphylococci into the abdominal wall of guinea-pigs previously treated with the staphylococcal vaccine was followed by a distinctly different response from that of normal guinea-pigs, as measured by the degree of illness, the fatality rate, the ability of the tissues to heal, the histologic reaction and the degree of phagocytosis. The degree of illness was never as pronounced in the previously treated guinea-pigs as in the normal ones. The fatality rate was one-half that of the normal guinea-pigs, and the ability of the tissues to heal after an area of necrosis was produced or after the excision of the area inoculated was surprisingly rapid and without complications. The histologic reaction was characterized by a localization of the staphylococci in clumps, usually in a small area of the subreticular layer, although in two instances this localization took place in the papillary layer. The epithelial covering of the area of localized cocci usually became necrotic. The central portion of the cellular reaction consisted of polymorphonuclear leukocytes and a few mononuclear cells. These polymorphonuclear and mononuclear phagocytes in turn were localized by innumerable mesenchymal cells of the local tissues. The phagocytic activity of the cells was marked, as evidenced by the great number of cocci within a single phagocyte.

The mechanism of localization of such large numbers of virulent staphylococci within a small area of skin of a locally treated guinea-pig as contrasted with their widespread dissemination in the skin of a normal guinea-pig, is obviously of great significance. Various ideas have been advanced to explain this mechanism. Cobbett and Melsome [9] thought that it was the result of an early inflammatory reaction, which destroyed the micro-organisms before they had time to adjust themselves to the new environment, to proliferate and to liberate their toxins. Wassermann and Citron [33] postulated the idea of "Umstimmung," or retuning,

32. Gay, F. P.; Clark, A. R., and Linton, R. W.: Arch. Path. **1**:857, 1926.

33. Wassermann, A., and Citron, J.: Deutsche med. Wchnschr. **31**:573, 1905.

of the cells of the area which had been sensitized. Noguchi [11] mentioned the possibility of a local formation of antibodies in the connective tissue of the areas previously in contact with a bacterial antigen. Opie [34] demonstrated the fixation of an antigen at the site of inoculation in tissues sensitized to that antigen. His explanation of such a phenomenon was based on the assumption of the local occurrence of an anaphylactic inflammation.

Menkin [35] recently advanced the idea that bacteria and particulate matter are fixed in an area previously inflamed because of the barrier action of thrombosed lymphatics and the deposition of fibrin. This explanation, however, seems inadequate in the present series of experiments, first, because the sections show many more patent than thrombosed blood vessels and lymphatics, and secondly, because of a complete absence of fibrin as revealed by the use of specific staining methods. Although there is a semblance of fibrin in sections of the skin of normal guinea-pigs inoculated with staphylococci and stained with hematoxylin and eosin, such is not the case in sections of the skin of guinea-pigs previously treated with the staphylococcal vaccine. Furthermore the use of nine specific stains for fibrin has failed in every instance to demonstrate its presence.

The countless numbers of phagocytes may act as a mechanical barrier, especially in cases of nonspecific immunity, but in specific immunity the local presence of immune bodies must be taken into consideration. It is probable that the general mechanism of fixation is as follows: The vaccine introduced intradermally is retained and metabolized principally by the local phagocytes, with the resulting formation of antibodies which in turn tend to fix the antigen at the site of inoculation by a precipitating reaction, as Opie [4] demonstrated, or else the union of antigen with antibody produces a physicochemical change in the tissues that hinders dissemination of the antigen from the area. Evidence for the presence of agglomerating or flocculating antibodies is furnished by the morphologic demonstration of agglutination in vivo, as reported in a previous paper by Cannon and Pacheco.[36] Further evidence of the presence of local antibodies is suggested by the great degree of swelling of the bacteria injected into the locally treated tissues and by the vigorous phagocytosis by the microphages and macrophages. Similar observations were made by Bordet [37] and Tsuda.[38] More recently, Cannon and Sullivan [39] demonstrated by methods of chemical extraction that anti-

34. Opie, E. L.: J. Immunol. **9**:255, 1924.

35. Menkin, V.: J. Exper. Med. **53**:171, 1931.

36. Cannon, P. R., and Pacheco, G. A.: Am. J. Path. **6**:749, 1930.

37. Bordet, J.: Ann. Soc. roy. d. sc. méd. et nat. de Brux. **4**:455, 1895.

38. Tsuda, S.: Virchows Arch. f. path. Anat. **247**:121, 1923.

39. Cannon, P. R., and Sullivan, F. L.: Proc. Soc. Exper. Biol. & Med. **29**:517, 1932.

bodies may be formed locally in an area of local immunization and may persist there in greater concentration than elsewhere, as, for instance, in adjacent unimmunized skin, blood serum, spleen and liver. These findings strengthen the view that local formation and concentration of antibodies may play an important part in the mechanism of local immunity. The "Umstimmung," or retuning, as suggested by Wassermann and Citron [33] may thus be explained as due to the local concentration of immune bodies formed by locally mobilized mesenchymal cells, the latter having elaborated the immune bodies during the period of local immunization. The introduction of antigen into a tissue containing locally a high concentration of specific antibodies between and probably on the surfaces of histiocytes may lead to an antigen-antibody reaction tending to localize the antigen immediately. Secondary to this union, the vasomotor reactions and leukocytic infiltration tend further to keep the antigen fixed, thus preventing the harmful effects of its generalization.

CONCLUSIONS

The continued local intradermal treatment of guinea-pigs with a heat-killed suspension of S. aureus leads to the proliferation and mobilization of large numbers of macrophages in the area treated.

The subsequent inoculation into such an area of living, virulent staphylococci is followed by an accelerated inflammatory response and healing, with localization of the bacteria in the area inoculated.

The localization is due primarily to an antigen-antibody reaction whereby the bacteria tend to become agglomerated and presumably opsonized, and then engulfed by phagocytes, both microphages and macrophages.

This localization does not seem to be significantly influenced by such mechanical barriers as a deposition of fibrin or thrombosis of lymphatics and blood vessels.

As a result of these local tissue changes the harmful effects of the generalization of the bacteria are to a great extent prevented.

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